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Non-Viral Delivery Systems in Gene Therapy and Vaccine Development

Azam Bolhassani and Sima Rafati

*Molecular Immunology and Vaccine Research Laboratory, Pasteur Institute of Iran, Tehran
Iran*

1. Introduction

Gene therapy is the process of treating a particular disease through the introduction of genetic material in order to elicit a therapeutic benefit [Stone, 2010]. The defective gene of a diagnosed patient can be corrected by a number of different strategies such as “gene replacement”, “gene correction”, and “gene augmentation” [Katare and Aeri, 2010]. In replacement therapy, a normal gene is inserted somewhere in the genome so that its product could replace that of a defective gene. This approach may be suitable for recessive disorders, which are marked by deficiency of an enzyme or other proteins. Although, the gene functions in the genome providing an appropriate regulatory sequence, the approach may not be successful in treating dominant disorders associated with the production of an abnormal gene product, which interferes with the product of normal gene [Katare and Aeri, 2010]. Corrective gene therapy requires replacement of a mutant gene or a part of it with a normal sequence. This can be achieved by using recombinant technology. Another form of corrective therapy involves the suppression of a particular mutation by a transfer RNA that is introduced into a cell [Katare and Aeri, 2010]. In gene augmentation, introducing a normal genetic sequence into a host genome modifies the expression of mutant gene in defective cell and the defective host gene remains unaltered. In general, the gene therapy recipient cells may be germline cells or somatic cells. Germline cells therapy involves modifying the genes in germ cells which will pass these genetic changes to the future generations. Somatic cells therapy involves the insertion of genes into specific somatic cells like the bone marrow stem cells, fibroblasts, hepatocytes or myocytes [Katare and Aeri, 2010]. This form of gene therapy is being used at most genetic engineering laboratories throughout the world.

Clearly, gene therapy provides great opportunities for treating diseases from genetic disorders, infections and cancer [Park et al., 2006]. While the genetic mutations underlying various diseases are well understood, delivering a corrective gene to the unhealthy organs/tissues remains a remarkable challenge [Stone, 2010]. To achieve successful gene therapy, development of proper gene delivery systems could be one of the most important factors. Gene delivery systems should be designed to protect the genetic materials from premature degradation in systemic blood stream and to efficiently transfer the therapeutic genes to target cells. Intracellular delivery systems will be required for all molecules that have intracellular function. For example, nucleic acid molecules including encoding genes, oligonucleotides and RNA molecules must enter cells and target the nucleus when transcription is the target. Regardless of the molecules for delivery, a common requirement

is the avoidance of endosomal uptake that may cause degradation and denaturation [Gould and Chernajovsky, 2007]. Several approaches are being developed that can be applied to the delivery of all these types of molecules at disease sites. For the goal to be fully achieved, cell-targeting strategies require still further development.

Currently, a number of older and more recently discovered techniques have been developed for therapeutic gene transfer. A variety of viral and non-viral possibilities are available for basic and clinical researches [Gardlik et al., 2005]. Among these studies, DNA based vaccines are becoming popular. They stimulate the CD4⁺ T cells of Th1 subset and thereby mediate cellular immune response, which is effective against pathogens. On the other hand, the recombinant protein vaccines stimulate Th2 subset of T cells thereby eliciting a humoral response. The studies showed that DNA vaccines have been successful in protecting animals against influenza, herpes, rabies, malaria and leishmaniasis [Katare and Aeri, 2010]. However, the potential disadvantages of DNA vaccine have reduced the value of the approach. To optimize antigen delivery efficiency as well as vaccine efficacy, the non-viral vector as vaccine carrier has shown particular benefits to avoid the obstacles that both peptide/protein and gene-based vaccines have encountered [Chen and Huang, 2005]. For example, the success of the liposome-based vaccine has been demonstrated in clinical trials and further human trials are also in progress. This chapter summarizes the non-viral delivery routes and methods for gene transfer used in gene therapy and vaccine development.

2. Gene delivery systems

The simplest way of gene delivery is injecting naked DNA encoding the therapeutic protein, but because of low efficiency, there is a need to use special molecules and methods to improve gene delivery. A vector can be described as a system fulfilling several functions, including (a) enabling delivery of genes into the target cells and their nucleus, (b) providing protection from gene degradation, and (c) ensuring gene transcription in the cell. The ideal DNA vehicle should also be suitable for clinical application. It has to be inexpensive and easy to produce and purify in large amounts [Gardlik et al., 2005]. Two kinds of vectors have been employed as vehicles for gene transfer: 1) Viral vectors for gene transduction (e.g., *retroviral*, *adenoviral*, *adeno-associated viral* and *lentiviral* vectors), and 2) Non-viral vectors for gene transfection based on lipids, water soluble polycations, non-condensing polymers and nano/ micro-particles [Gardlik et al., 2005; Katare, 2010]. However, each vector has its own advantages and disadvantages.

2.1 Viral vectors (biological delivery systems)

Viral techniques use various classes of viruses as a tool for gene delivery [Gardlik et al., 2005; Stone, 2010]. Viruses introduce their DNA into the cells with high efficiency. Therefore, it is possible to take advantage of this system by introducing a foreign gene into the virus and then using the properties of the virus to deliver this gene with high efficiency into the target cells [Gardlik et al., 2005]. Gene therapy vectors are being developed by genetic modification of *retroviruses*, *adenoviruses*, *poxviruses*, *parvoviruses* (*adeno-associated viruses*), *herpesviruses* etc. [Gardlik et al., 2005; Stone, 2010]. Unlike wild type viruses, these vectors are used to transfer therapeutic genes into target cells and thus are engineered by deleting the essential genes which allow replication, assembling or infection. Replication deficiency ensures the safety of viral vectors, but on the other hand, vectors need to be

produced in large amounts of virus particles. For this purpose, there are specialized cell lines called “packaging cell lines” (PCLs) engineered to replace a function of a deleted viral gene and for the production of recombinant viruses [Gardlik et al., 2005]. However, the interaction between a vector and a host-cell genome cannot be completely eliminated. Some disadvantages of viral delivery are addressed in Table 1.

No.	Disadvantage
1	Generation of an immune response to expressed viral proteins that subsequently kill the target cells producing a therapeutic gene product
2	Random integration of some viral vectors into the host chromosome
3	Clearance of viruses delivered systemically
4	Difficulties in engineering viral envelopes or capsids to achieve specific delivery to cells other than those with natural tropism for the virus
5	Possible recombination of the viral vector with DNA sequences in the host chromosome that generates a replication-competent, infectious virus
6	Inability to administer certain viral vectors more than once
7	High costs in producing large amounts of high-titer viral stocks for use in the clinic
8	Limited size of the nucleic acid that can be packaged and used for viral gene therapy

Table 1. Viral delivery system disadvantages [Templeton and Lasic, 1999; Gupta et al., 2004]

Currently, developed Viruses as transfer vectors are divided into two classes, following their different strategies for replication and survival: a) Non-lytic viruses, including *retroviruses* and *lentiviruses*, produce virions from the cellular membrane of an infected cell, leaving the host cell relatively intact; b) Lytic viruses, including human *adenovirus* and *herpes simplex virus* families, destroy the infected cell after replication and virion production. This native nature of the original viruses determines the use of each recombinant replication-defective viral vector in clinical applications [Table 2]. Despite some limitations on the use of viral vectors regarding safety and reproducibility, they are still the most used gene transfer vehicles [Gardlik et al., 2005; Katare, 2010; Gupta et al., 2004].

Vector	Genome	Structure	Properties
<i>Adenoviruses</i>	dsDNA	Capsid	Transient expression, strong immunogenicity
<i>Alphaviruses</i>	RNA	Envelope	Transient, but extreme, expression levels; low immunogenicity
<i>HSV</i>	dsDNA	Envelope	Latent infection, long-term expression, low toxicity (mutant)
<i>Lentiviruses</i>	RNA	Envelope	Genome integration, long term expression, safety concerns low titers, inefficient production
<i>Retroviruses</i>	RNA	Envelope	Genome integration, long-term expression
<i>Adeno-associated viruses (AAV)</i>	ssDNA	Capsid	Slow expression onset, genome integration, long term expression, inefficient large-scale virus production

Table 2. Utilization of viral vectors for gene delivery [Katare and Aeri, 2010]

In clinical studies, a recombinant *vaccinia* virus vector has been developed to express single or multiple T cell co-stimulatory molecules as a vector for local gene therapy in patients with malignant melanoma. This approach generated local and systemic tumor immunity and induced effective clinical responses in patients with metastatic disease [Kim-Schulze and Kaufman, 2009]. Furthermore, PSA-TRICOM vaccine (prostate-specific antigen plus a TRIad of co-stimulatory molecules; PROSTVAC) includes a priming vaccination with recombinant *vaccinia* (rV)-PSA-TRICOM and booster vaccinations with recombinant fowlpox (rF)-PSA-TRICOM. Each vaccine consists of the transgenes for PSA, including an agonist epitope, and three immune co-stimulatory molecules (B7.1, ICAM-1, and LFA3; designated TRICOM) [Kaufman, 2002]. The efficacy of PSA-TRICOM has been evaluated in phase II clinical trials in patients with metastatic hormone-refractory prostate cancer (mHRPC). PANVAC-VF, another poxviral-based vaccine, consists of a priming vaccination with rV encoding CEA (6D), MUC1 (L93), and TRICOM plus booster vaccinations with rF expressing the identical transgenes. CEA (6D) and MUC1 (L93) represent carcinoembryonic antigen and mucin 1 glycoprotein, respectively, with a single amino acid substitution designed to enhance their immunogenicity. This vaccine is currently under evaluation in several different types of CEA or MUC1-expressing carcinomas and in patients with a life expectancy more than three months [Vergati et al., 2010].

2.2 Non-viral vectors (Non-biological gene delivery systems)

In comparison with virus-derived vectors, non-viral vectors have several advantages, such as the safety of administration without immunogenicity, almost unlimited transgene size and the possibility of repeated administration [Gardlik et al., 2005]. Non-viral gene delivery systems generally consist of three categories: (a) naked DNA delivery, (b) lipid-based and (c) polymer-based delivery [Park et al., 2006]. Therapeutic gene can be introduced into the target cell either as an insert in plasmid with regulation sequences, what enables the regulation control of expression (inducible promoter) or as a PCR product. The simplest way of gene introduction is an injection of naked DNA into target cells. Such a naked plasmid DNA was used in several pre-clinical and clinical trials [Gardlik et al., 2005]. For example, some positive results were gained in cancer therapy by intra-tumoral injection of tumor suppressor genes or cytokines. However, this approach does not have the transfection efficiency of viral vectors. Low transfection efficacy and short-term expression still remain the main disadvantages of naked DNA gene transfer compared with viral vectors. Thus, many techniques have been developed to improve the introduction of therapeutic gene. Physical methods like electroporation and gene gun increase the entry of transgene into target cells. In addition, chemical methods like lipoplexes (DNA-liposomes complexes) and polyplexes (DNA-polymers complexes) improve the stability of DNA and also facilitate the entry into the cell [Gardlik et al., 2005]. Main methods of gene delivering systems are summarized in figure 1.

3. Physical delivery systems

A number of methods utilizing various physical techniques have been developed to facilitate the transfer of foreign genes into the host cells [Katare and Aeri, 2010]. Among them, electroporation and gene gun are further involved in preclinical and clinical trials.

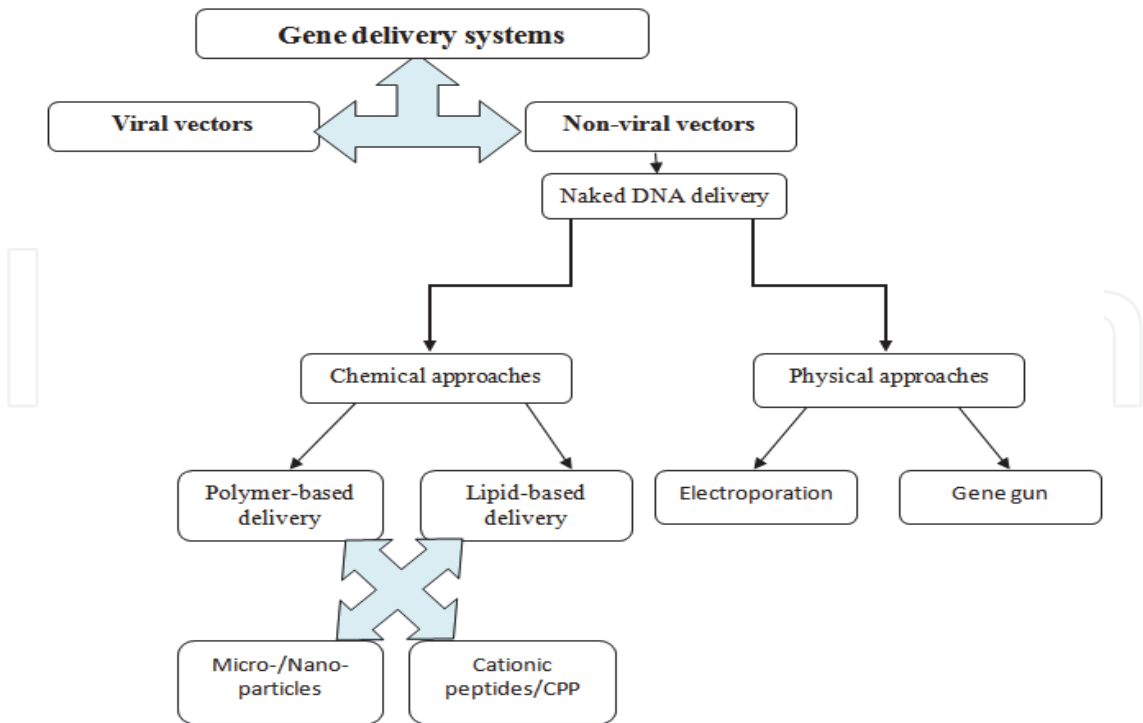


Fig. 1. Summary of the main methods of gene delivery systems

3.1 Electroporation

One of the methods that improve DNA penetration of the cell is electroporation [Lee et al., 2009; , Harrison et al., 1998; Rossini et al., 2002; Ahmad et al., 2009; Collins et al., 2006; Kang et al., 2011;]. *In vivo* use of electroporation is done by injecting naked DNA followed by electric pulses from electrodes that are located *in situ* in the target tissues. Successful use of electroporation was observed in transfecting muscles, brain, skin, liver, and tumors [Gardlik et al., 2005; Garcia-Frigola et al., 2007; Umeda et al., 2004; Babiuk et al., 2006; Harrison et al., 1998; Kang et al., 2011]. Since every tissue is specific and has its own characteristics, there are no generally accepted optimal conditions of electroporation that are suitable for effective transfection. These are dependent both on the amplitude and duration of the electric pulses and on the amount and concentration of DNA [Gardlik et al., 2005]. The generated pulse may be either a high voltage (1.5 kV) rectangular wave pulse for a short duration or a low voltage (350 V) pulse for a longer duration [Katare and Aeri, 2010].

Up to now, several clinical trials have been planned using the electroporation with DNA vaccines for cancer therapy such as: a) Intra-tumoral IL-12 DNA plasmid (pDNA) [ID: NCT00323206, phase I clinical trials in patients with malignant melanoma]; 2) Intratumoral VCL-IM01 (encoding IL-2) [ID: NCT00223899; phase I clinical trials in patients with metastatic melanoma]; 3) Xenogeneic tyrosinase DNA vaccine [ID: NCT00471133, phase I clinical trials in patients with melanoma]; 4) VGX-3100 [ID: NCT00685412, phase I clinical trials for HPV infections], and 5) IM injection prostate-specific membrane antigen (PSMA)/pDOM fusion gene [ID: UK-112, phase I/II clinical trials for prostate cancer] [Bodles-Brakhop and Draghia-Akli, 2008; Bodles-Brakhop et al., 2009].

Furthermore, Hepatitis C virus DNA vaccine showed acceptable safety when delivered by Inovio Biomedical's electroporation delivery system in phase I/II clinical study at Karolinska University Hospital. ChronVac-C is a therapeutic DNA vaccine being given to

individuals already infected with hepatitis C virus with the aim to clear the infection by boosting a cell-mediated immune response against the virus. This vaccination was among the first infectious disease DNA vaccine to be delivered in humans using electroporation-based DNA delivery [Bodles-Brakhop et al., 2009].

3.2 Gold bullet/gene gun

Similar to electroporation, another method, called “gene-gun”, does not require the presence of complicated and potentially toxic delivery systems [Gardlik et al., 2005]. The gene transfer is mediated by small particles of gold on which the DNA is bounded. These particles are then shot into the cell under great pressure and speed (with the help of compressed helium) and so pass the membrane barrier [Katare and Aeri, 2010]. At first, the gene-gun was developed for gene transfer into plant cells; then, its use has expanded to gene transfer into the mammalian cells. Effective development of the gene-gun was also achieved in the field of DNA vaccination. The latest clinical experiments focus on cancer vaccines against various human tumors [Gardlik et al., 2005]. This method has been successfully used to deliver DNA *in vivo* into liver, skin, pancreas, muscle, spleen and tumors. Expression of reporter genes (e.g. firefly luciferase and β -galactosidase) or therapeutic genes (human growth hormone) have also been reported by this method [Gardlik et al., 2005]. Recently, gene gun-mediated transgene delivery system has been used for skin vaccination against melanoma using tumor-associated antigen (TAA) human gp100 and reporter gene assays as experimental systems [Aravindaram and Yang, 2009 S]. In addition, the delivery of HPV DNA vaccines using intradermal administration through gene gun was shown to be the most efficient method of vaccine administration in comparison with routine intramuscular injection [Ogris and Wagner, 2002].

Currently, a HPV16 DNA vaccine encoding a signal sequence linked to an attenuated form of HPV16 E7 (E7 detox) and fused to heat shock protein 70 [(Sig/E7detox/HSP70)] has been used in clinical trials. In a previous study, the immunologic and anti-tumor responses have been evaluated by the pNGVL4a-Sig/ E7 (detox)/ HSP70 vaccine administered using three different delivery methods including needle intramuscular, biojector and gene gun. According to obtained results, DNA vaccine administered via gene gun generated the highest number of E7-specific CD8⁺ T cells as compared to needle intramuscular and biojector administrations in mice model [Trimble et al., 2003].

4. Chemical delivery systems

In order to facilitate the effective transfer of non-viral DNA into the cells, synthetic vectors improving the DNA admission into the cell and protecting it from undesirable degradation were designed. The most chemical delivery systems were derived from lipids or synthetic polymers [Templeton and Lasic, 1999].

4.1 Lipoplexes (cationic lipids/liposomes)

Plasmid DNA can be covered by lipids into organized structures such as liposomes or micelles [Templeton and Lasic, 1999]. The complex of DNA with lipids is called lipoplex. Lipoplexes can be divided into two types: 1) Anionic and neutral liposomes: At first, these kinds of lipids were used for the construction of synthetic vectors. Although, they were characterized by safety, compatibility with body fluids and the possibility of tissue-specific gene transfer, but the level of transduced cell expression was relatively low. At present, new

neutral and anionic liposomes suitable for *in vivo* gene therapy are being constructed [Gardlik et al., 2005; Gupta et al., 2004]; 2) Cationic liposomes: These lipids are naturally produced complexes with negatively charged DNA. Moreover, their positive charge allows interactions with the negatively charged cell membrane and thus penetration into the cell is permitted [Gardlik et al., 2005]. Cationic liposomes ensure effective protection against the degradation of the foreign DNA by the cell. The interactions of liposomes with DNA and the subsequent lipoplex formation are dependent on several physical conditions (pH, charge) as well as structural characteristics of the liposomes. The most frequent use of DNA-liposome complexes is in gene transfer into cancer cells, where the applied genes stimulate anti-tumor immune responses or genes decreasing the activity of oncogenes [Gardlik et al., 2005]. Recent studies revealed the ability of lipoplex gene transfer into the epithelial cells of the respiratory tract, which supports their usage in the therapy of respiratory diseases and cystic fibrosis. Their expression in all main organs, mostly in lungs, was observed after intravenous administration of lipoplexes. Targeted transfection can be gained, to some extent, by the addition of tissue-specific target ligand. It is suggested that the transfection is based on endocytosis of the host cell [Gardlik et al., 2005].

The advantages of using liposomes for gene therapy are included as: 1) lack of immunogenicity; 2) lack of clearance by complement system using improved formulations; 3) unlimited size of nucleic acids that can be delivered, from single nucleotides up to large mammalian artificial chromosomes containing several thousand kilobases; 4) ability to perform repeated administrations *in vivo* without adverse consequences; 5) low cost and relative ease of generating nucleic acid: liposome complexes that deliver therapeutic gene products in large scale; 6) safety, because plasmids used for non-viral delivery contain no-viral sequences, thereby precluding generation of an infectious virus; 7) naked DNA carried by liposome increases its uptake by antigen-presenting cells (APCs); 8) naked DNA carried by liposome enhances both humoral and cellular immunity; 9) naked DNA carried by liposome induces cytotoxic T lymphocyte response [Trimble et al., 2003].

For vaccine development, a general overview of different lipid-based particulate delivery systems, their composition, preparation methods, typical size, route of administration and model antigens has been listed by Myschik J. *et al.*, 2009 [Myschik et al., 2009]. Stimuvax (BLP25 liposome vaccine, L-BLP25, Oncothyreon partnered with Merck KGaA) is a cancer vaccine designed to induce an immune response against the extracellular core peptide of MUC1, a type I membrane glycoprotein widely expressed on many tumors (i.e., lung cancer, breast cancer, prostate cancer and colorectal cancer) [Vergati et al., 2010]. Stimuvax consists of MUC1 lipopeptide BLP25 [STAPPAHGVTSAPDTRPAPGSTAPPK (Pal) G], an immunoadjuvant monophosphoryl lipid A, and three lipids (cholesterol, dimyristoyl phosphatidylglycerol, and dipalmitoyl phosphatidylcholine), capable of enhancing the delivery of the vaccine to APCs. A randomized phase II B clinical trial evaluated the effect of Stimuvax on survival and toxicity in 171 patients with stage III B and IV non-small cell lung cancer (NSCLC), after stable disease or response to first-line chemotherapy. Based on these data, Merck is currently conducting three large phase III clinical trials of Stimuvax. This study will involve more than 1300 patients [Vergati et al., 2010].

Furthermore, a cationic lipid DNA complex (CLDC) consisting of DOTIM/cholesterol liposomes and plasmid DNA, containing immunostimulatory CpG and non-CpG motifs has been designed, with potential immunostimulating and anti-neoplastic activities. Upon systemic administration, TLR-directed cationic lipid-DNA complex JVRS-100 enters dendritic cells (DCs) and macrophages; immunostimulatory DNA binds to and activates

Toll-like receptors (TLRs), which may result in the generation of anti-tumor natural killer (NK) cell and T-cell responses by the innate immune system. In addition, as a vaccine adjuvant, this agent may induce a strong cytotoxic T-lymphocyte (CTL) response to co-administered antigen. The efficacy of JVRS-100 has been evaluated in phase I clinical trials for the treatment of patients with Relapsed or Refractory Leukemia [ID: NCT00860522].

4.2 Polyplex (polysaccharides/cationic polymers)

A wide range of polymeric vectors have been utilized to deliver therapeutic genes *in vivo*. The modification of polymeric vectors has also shown successful improvements in achieving target-specific delivery and in promoting intracellular gene transfer efficiency [Park et al., 2006, Ogris and Wagner, 2002]. Various systemic and cellular barriers, including serum proteins in blood stream, cell membrane, endosomal compartment and nuclear membrane, were successfully avoided by designing polymer carriers having a smart molecular structure [Park et al., 2006; Ogris and Wagner, 2002]. Vectors based on a complex of polymers with DNA are called polyplexes. Most of them consist of cationic polymers and their production is regulated by ionic interactions [Gardlik et al., 2005].

In contrast to lipoplexes, some polyplexes (polylysine) are not able to release intracellular DNA into the cytoplasm [Gardlik et al., 2005]. For this purpose, co-transfection with endosome-lytic agents (inactivated *adenovirus*) is needed. On the other hand, polymers such as polyethylenimine have a mechanism of endosome disruption and there is thus no need for transfection with endosome-lytic agents. Polyethylenimine is used as a vector in aerosol inhalation gene therapy. It is a non-invasive and relatively effective gene transfer, especially into the respiratory tract, with permanent gene expression in this target region without undesirable expression in other tissues [Gardlik et al., 2005]. The size of the polymer determines the transfection efficiency and is specific for each individual gene transfer. Furthermore, the size of the aerosol particles determines the place of action, and thus the specificity of inhalation gene therapy [Gardlik et al., 2005]. An alternative to polyplexes can also be the use of polymer nanoparticles. Two types of such complexes have been characterized as gelatin-DNA and chitosan-DNA. In comparison with naked DNA, transfection using nanoparticles shows increased expression *in vivo* when administrated intratracheally or intramuscularly. Some polymeric vectors are summarized in the following sections:

Poly (L-lysine) (PLL)-based gene delivery systems

PLL has been widely used as a non-viral gene carrier since the formation of polyelectrolyte complexes between PLL and DNA was identified [Park et al., 2006]. Although, PLLs with high molecular weight have some properties suitable for a gene carrier, the PLL/DNA complexes showed a relatively high cytotoxicity and a tendency to aggregate and precipitate depending on the ionic strength. PEGylation [PEG: polyethylene glycol] of cationic polymers is known to greatly improve the problems of cytotoxicity, aggregation and non-specific protein adsorption *in vivo* [Park et al., 2006]. Some examples are mentioned as following:

- **Sugar-conjugated PLL:** Lactose and galactose have been used as conjugation partners with polymeric gene carriers for targeting asialoglycoprotein of hepatocytes. Lac-PEG-PLL showed much less cytotoxicity and higher stability and solubility in a physiological condition compared to PLL [Park et al., 2006].

- **Arterial-wall binding peptide (AWBP)-conjugated PLL:** Arterial-wall binding peptide (AWBP) is a peptide containing the arterial-wall binding domain (1000-1016 amino acids) of apoB-100 protein, a major protein component of LDL. The AWBP was conjugated to PLL via a PEG linkage (AWBP-PEG-g-PLL). When interacted with a plasmid DNA, AWBP-PEG-g-PLL could form spherical shaped complexes with a size of 100 nm and showed dramatic increase of transfection efficiency (150-180-fold), compared to PLL and PEG-g-PLL, in bovine aorta endothelial cells and smooth muscle cells. The presence of free AWBP in the transfection medium reduced the transfection efficiency of AWBP-PEG-g-PLL, suggesting that AWBP-PEG-g-PLL could be used as a tissue-selective gene carrier [Park et al., 2006].
- **Antibody-PLL conjugates:** Antibody-antigen interaction is one of the most specific interactions in biological systems. A monoclonal antibody against leukemia-specific JL-1 antigen (anti-JL-1-Ab) was conjugated with PLL by periodate-mediated oxidation of carbohydrate moiety in the Fc domain of the antibody, followed by reaction with PLL. The anti-JL-1-Ab-PLL conjugate demonstrated significantly higher transfection efficiency than PLL or lipofectin in leukemia (Molt 4) cells [Park et al., 2006].
- **Folate-conjugated PLL:** Folate receptor has been identified as a potential target molecule of various cancer cells. The receptor is up-regulated and over-expressed in a number of rapidly growing malignant tumor cells, resulting in a dramatic promotion of the cellular uptake of folate. Therefore, the conjugation of folate to a variety of polymeric carriers has been chosen as a popular strategy for the target-specific delivery of anti-cancer therapeutics to the folate receptor-bearing tumor cells. A folate-PLL conjugate that incorporates a PEG spacer between folate and PLL (Fol-PEG-PLL) was also synthesized. The Fol-PEG-PLL was coated onto the complexes of PEI/DNA for a receptor-mediated gene transfer. The formulated complexes exhibited much higher transfection efficiency than PEI/DNA or lipofectamine/DNA complexes in the presence of 10% serum, suggesting that the PEG segment of Fol-PEG-PLL could increase the cellular uptake by receptor-mediated endocytosis and efficiently stabilize the complexes by increasing their solubility as well as by reducing the non-specific adsorption of serum proteins. The formulation also showed much lower cytotoxicity than PEI/DNA complexes [Park et al., 2006].
- **The Terplex system:** The Terplex system is a low-density lipoprotein (LDL)-mediated targeting system, where the LDL specifically interacts with the LDL receptors on the cell surface. LDL receptors are membrane-anchored proteins present in many cell types including hepatocytes, endothelial cells and myocytes. The stearyl-PLL conjugate synthesized by N-alkylation of PLL with stearyl bromide interacts with a plasmid DNA to form complexes. The stearyl group could then bind to LDL via hydrophobic interaction to form the supramolecular gene carrier, the terplex. The Terplex system showed efficient *in vitro* transfection in a variety of cells including smooth muscle cells (A7R5), and human lung fibroblasts (CCD-32 Lu). The systemic administration of the Terplex system demonstrated prolonged circulation time compared to naked DNA [Park et al., 2006].

Polyethylenimine (PEI)-based gene carriers

- PEI has been one of the most popularly employed cationic gene carriers due to its superior transfection efficiency in many different types of cells. The buffering property of PEI leads to protect the DNA from degradation in the endosomal compartment

during the maturation of the endosome to lysosome, facilitating intracellular trafficking of DNA. High cation density of PEI also contributes to the formation of highly condensed particles by interacting with DNA. However, the property may confer significant cytotoxicity. Studies with linear PEIs showed even higher transfection efficiency and lower cytotoxicity compared to branched PEI [Park et al., 2006]. There are different spectra utilizing PEI as described in following sections:

- **PEI-PEG:** PEI-grafted PEGs (PEI-g-PEG) with different PEG grafting ratios were synthesized to address the cytotoxicity and aggregation problems of PEI. Cell cytotoxicity of PEI-g-PEG was greatly reduced, while the transfection efficiency of PEI-g-PEG was still comparable to that of PEI. Cytotoxicity was independent of molecular weight of PEG but affected by the degree of PEG substitution [Park et al., 2006].
- **PEI conjugates with targeting moieties:** a) PEI-g-PEG-RGD: An angiogenic endothelial cell targeted gene delivery system (PEI-g-PEG-RGD) was developed by incorporating the $\alpha v\beta 3/\alpha v\beta 5$ integrin binding RGD peptide. b) Antibody-conjugated PEIs: A monoclonal antibody against human epidermal growth factor receptor-2 (HER-2) was conjugated to linear PEI for targeted gene transfer to cancer cells. The HER-2 antibody-PEI conjugate showed enhanced transfection efficiency in HER-2 over-expressing human breast adenocarcinoma cells (Sk-Br-3) compared to unmodified PEI [Park et al., 2006].
- **Folate-conjugated PEIs:** Folate-polyethylene glycol-folate-grafted-polyethylenimine (FPF-g-PEI) was synthesized by grafting folate-PEG-folate to PEI. A PEI-PEG-folate (PEI-PEG-FOL) conjugate was used as a carrier for a plasmid encoding small interfering RNA (siRNA) targeting green fluorescence protein (GFP). The complexes between the PEI-PEG-FOL and the siRNA-expressing plasmid showed an efficient suppression of GFP expression compared to unmodified PEI complexes in folate receptor over-expressing cells (KB), which stably expressed GFP [Park et al., 2006].

Water-soluble lipopolymer (WSLP): The water-soluble lipopolymer (WSLP) was synthesized by conjugating cholesteryl chloroformate to a low molecular weight PEI (1.8 kDa). WSLP interacts with DNA to form stable colloidal particles (70 nm). The PEI moiety of WSLP confers a buffering effect, which could facilitate endosomal escape of the WSLP/DNA complex. It was also reported that the dodecylation of PEI enhanced the cellular uptake and transfection efficiency. In a similar way, the hydrophobic cholesterol moiety of WSLP would give a chance to form small and stable complexes, resulting in enhanced cellular uptake and transfection efficiency. WSLP showed higher transfection efficiency and much lower cytotoxicity than 25 kDa PEI, suggesting that WSLP has the advantages from PEI as well as from cholesterol. Intratumoral injection of WSLP/p2CMVmIL-12 complexes to tumor-bearing mice showed a significant improvement in the retardation of tumor growth and survival rate [Park et al., 2006].

Biodegradable polycations

The backbone linkages of most polymeric gene carriers consist of a -C-C- bond or amide bond, which are not degraded in physiological solutions. The non-degradable non-viral carriers are not easily removed by physiological clearance systems and therefore, can possibly accumulate within cells or tissues to elicit further cytotoxicity [Park et al., 2006]. To solve the problems, several biodegradable polycations have been synthesized and evaluated as potential gene carriers. Generally, the biodegradable polycations showed much less cytotoxicity and higher transfection efficiency compared to an unmodified polycations, such

as PLL or PEI [Park et al., 2006]. Some examples include: Poly (α -[4-aminobutyl]-L-glycolic acid) (PAGA), a biodegradable PLL analogue; Poly(β -amino ester)s; Poly(2-aminoethyl propylene phosphate) (PPE-EA); Degradable PEIs; The biodegradable PEIs were synthesized by crosslinking low molecular weight PEI (0.8 kDa) with either PEG-bis-succinimidyl succinate or disulfide-containing cross-linkers, such as dithiobis (succinimidylpropionate) (DSP) and dimethyl 3,3'-dithiobispropionimidate (DTBP) [Park et al., 2006].

Neutral and non-condensing polymer-based gene delivery systems

Although, cationic polymers, which electrostatically interact with DNA to neutralize its negative charge and condense DNA into nanosized particles, are generally considered as gene carriers, neutral polymers such as polyvinyl alcohol (PVA) and polyvinyl-pyrrolidone (PVP) can also be used for gene transfer. They can protect DNA from enzymatic degradation and facilitate cellular uptake of DNA. These polymers may interact with DNA via hydrogen bonding and/or van der Waals interactions; van der Waals interaction of the hydrophobic vinyl backbones may cover around DNA to make its surface more hydrophobic. In a study, intramuscularly administered PVP/plasmid DNA formulation resulted in a significant increase in the number and distribution of the reporter-gene expressing cells in rat tibialis, compared to naked plasmid [Park et al., 2006].

5. Micro-/nano-particles

Another approach to DNA-vaccine delivery involves microparticle-based technologies to target APCs [Ulmer et al., 2006]. Microencapsulation of DNA, or association of DNA with microcapsules, has led to enhancement of CTL responses to encoded proteins [Doria-Rose and Haigwood, 2003]. Biodegradable, non-antigenic poly-lactide polyglycolide (PLGA or PLG) microspheres offer many advantages as a vaccine delivery system. Both cellular and humoral immune responses can be elicited to antigens encapsulated in, or conjugated onto PLG microspheres. Particles used typically range in size from 1 to 10 μm in diameter, a size that is readily phagocytosed by dendritic cells and other antigen-presenting cells (APCs). Microspheres elicit both CD8⁺ and CD4⁺ T cell responses by releasing antigen intracellularly [Doria-Rose and Haigwood, 2003]. Biodegradable PLGA nanoparticles (NPs) have been investigated for sustained and targeted/localized delivery of different agents, including drugs, proteins and peptides and recently, plasmid DNA owing to their ability to protect DNA from degradation in endolysosomes. PLGA-based nanotechnology has been widely used in diagnosis and treatment of cancer. These NPs have been shown to stimulate the immune response as measured by an increase in IL-2 and IFN- γ in spleen homogenates [Lu et al., 2009]. The majority of the existing literature involving PLGA polymers has tended to be focused on PLGA microspheres. In the last 10 years, microspheres have been used extensively for the injectable delivery of vaccine antigens, both for viral and bacterial antigens. Similar to microspheres, PLGA NPs have been shown to effectively enhance immune responses. The major obstacle is providing delivery vehicles with the adequate surface molecules for recognition by the immune system and for more effective targeting. It is likely, therefore, that future studies of PLGA NPs as vaccine candidates will focus on improving these features, as recently tested by grafting RGD peptides (arginine-glycine-aspartic acid-containing synthetic peptides) covalently onto PEG moieties on the surface of PLGA NPs [Lu et al., 2009].

These polymers have been designated as feasible candidates for drug delivery systems, anti-cancer agents and vaccine immunotherapy. For example, DNA vaccine delivery to APCs has been facilitated by microencapsulation of plasmid DNA, which encodes HPV E6/E7 antigenic proteins. The capsule is formed from polymeric PGLA microparticles. These resulting microparticles have a greater propensity toward APC uptake compared to naked DNA. This technique allows HPV DNA plasmid to be condensed inside the microparticle. The physical and chemical properties of the PGLA scaffold make DNA inaccessible to nuclease and preventing degradation, allowing for a sustained release of DNA and enhancing transfection efficiency *in vitro* [Lin et al., 2010]. In mice, microspheres containing HPV plasmid encoding HPV E6/E7 antigens have been shown to elicit a strong antigen-specific cytotoxic T cell response. Using this technology, microencapsulated DNA vaccine termed ZYC-101 encoding multiple HLA-A2 restricted HPV E7 epitopes has undergone Phase I trials in patients with CIN2/3 lesions and high-grade anal intraepithelial neoplasia. In both trials, intramuscularly administered vaccine was well tolerated, and in some patients had resulted in histological regression of the lesions as well as generation of E7-specific IFN- γ expressing T cells. A newer version of the DNA vaccine, ZYC-101a, which encodes HPV16 and HPV18 E6- and E7-derived epitopes has been used in phase II clinical trial in patients with CIN 2/3 lesions [Lin et al., 2010].

The multi-functional nano-devices based on the dendritic polymer or dendrimers are also being applied to a variety of cancer therapies to improve their safety and efficacy. Technical advances have been focused on the development of a linking strategy that allows the dendrimer molecules to be linked via complementary oligonucleotides. At present, further applications of dendrimers in photodynamic therapy, boron neutron capture therapy, and gene therapy for cancer are being examined [Baker, 2009].

Recently, the modified fluorescent nanoparticles have been synthesized as a targeting and delivery system, by conjugating both tumor targeting agent and chemokines to the nanoparticles, in order to attract immune cells toward tumor cells. Biodegradable chitosan nanoparticles encapsulating quantum dots were prepared, with suitable surface modification to immobilize both tumor targeting agent and chemokine on their surfaces [Chatterjee and Zhang, 2007]. Fluorescent chitosan coated quantum dots (QDs) were used to act as bi-functional bridging units between cancer and immune cells. This nanoparticulate form of delivery promises the advantages of enhanced tumor selectivity and longer half-lives, thereby enhancing effectiveness of the immune response and reduction in systemic toxicity [Chatterjee and Zhang, 2007]. Furthermore, the rapid development of Quantum Dots (QDs) technology has already fulfilled some of the hopes of developing new, more effective cancer-imaging probes. First, stable encapsulation of QDs with amphiphilic polymers has prevented the quenching of QD fluorescence in the aqueous *in vivo* environment. Second, QDs are relatively inert and stable. Finally, successful conjugation of QDs with biomolecules has probably made active targeting them to tumors. Despite their success so far in cancer imaging, there are challenges in enhancing sensitivity, maximizing specificity and minimizing toxicity of QDs, which must be undertaken before clinical applications can proceed [Zhang et al., 2008]. A major parameter limiting immune responses to vaccination is the number of activated APCs that capture antigen and migrate to draining lymph nodes. The use of cellular magnetic resonance imaging (MRI) is a promising approach for this purpose [Long et al., 2009]. In a study, an *in vivo* labeling method was described, which relies upon cell-to-cell transfer of super-paramagnetic iron oxide (SPIO) from tumor cells to endogenous APCs, *in situ*, for quantification of APC delivery to lymph

nodes in a tumor vaccine model. Mice were immunized with a tumor cell-based vaccine that was labeled with SPIO. APCs that had captured SPIO were imaged over time as they accumulated in lymph nodes. It was indicated that MRI is capable of monitoring, *in vivo*, the trafficking of magnetically labeled APCs inducing a tumor-specific immune response, and that these cells can be magnetically recovered *ex vivo*. Excellent correlation was observed between *in vivo* and *ex vivo* quantification of APCs, with resolution sufficient to detect increased APC trafficking elicited by an adjuvant [Long et al., 2009].

6. Cationic peptides/cell-penetrating peptides (CPP)/trojan peptides

The studies have shown that a number of peptides and proteins are able to penetrate the cell membrane and enter the cell. It has been observed that many cargo molecules that are covalently attached to these peptides will be translocated into the cell. Recently, various natural and/ or synthetic cell-penetrating peptides (CPP) have known as efficient tools in vaccine design as they are capable of delivering therapeutic targets into cellular compartments. In fact, the cell membrane is impermeable to hydrophilic substances and delivery into cells could be facilitated by linking to CPP. Different cargos such as drugs, peptide/ protein, oligonucleotide/ DNA/ RNA, nanoparticles, liposomes, bacteriophages, fluorescent dyes and quantum dots have been linked to CPPs for intracellular delivery with possible use in future vaccine design [Brooks et al., 2010]. Two applications of CPP already validated in vaccine studies are delivery of tumor-associated antigens into antigen-presenting cells (APCs) and use as a non-viral gene delivery vehicle in DNA vaccines. There are two methods for designing CPP incorporating immunogenic antigens: A) chemical linking via covalent bonds B) coupling via recombinant fusion constructs produced by bacterial expression vectors. The orientation of the peptide and cargo and the type of linkage are likely important [Brooks et al., 2010]. In addition, the utilized CPP, attached cargo, concentration and cell type, all significantly affect the mechanism of internalization. The mechanism of cellular uptake and subsequent processing still remains controversial. It is now apparent that CPP mediate intracellular delivery via both endocytic and non-endocytic pathways [Brooks et al., 2010; Jarver and Langel, 2004; Wagstaff and Jans, 2006]. An attractive feature of using polypeptides as gene delivery vectors is incorporating multiple functional domains into one polypeptide chain, such as a DNA-binding domain linked with a receptor-targeting domain. This kind of polypeptides will recognize and bind to cell surface receptors that are unique to target cells and deliver the bound DNA into the cells through receptor-mediated endocytosis. Therefore, this process may ensure the therapeutic effect in desired cells and limit the potential side effects caused by transgene expression into the non-targeted cells [Zeng and Wang, 2005].

Several studies have shown that oligo-deoxynucleotides (ODN) with immune-stimulating sequences (ISS) containing CpG motifs facilitate the priming of MHC class I- restricted CD8+ T cell responses to proteins or peptides. Therefore, ODN/cationic peptide complexes are potent tools for priming CD8+ T cell immunity [Schirmbeck et al., 2003]. The complex formation required electrostatic linkage of the positively charged peptide to the negatively charged ODN. Conjugation of immunostimulatory DNA or ODN to protein antigens facilitates the rapid, long-lasting, and potent induction of cell-mediated immunity. It was shown that ODN (with or without CpG-containing sequences) are potent Th1-promoting adjuvants when bound to cationic peptides covalently linked to antigenic epitopes, a mode of antigen delivery existing in many viral nucleocapsids [Schirmbeck et al., 2003]. Table 3

contains a list of peptides that have been investigated for their ability to penetrate the cell [Futaki, 2005; Brooks et al., 2010].

Class	Protein	Functional peptide	Sequence	Translocation efficiency	Reference
Basic	TAT [HIV]	Tat and related peptides			Vives et al., 1997
		HIV-1 Tat (48-60)	GRKKRRQRRRPPQ	+++	Futaki, 2005; Brooks et al., 2010
		R9-Tat	GRRRRRRRRRPPQ	+++	Futaki, 2005
Basic		Arginine-rich RNA binding peptides			Futaki, 2005; Brooks et al., 2010
		HIV-1 Rev-(34-50)	TRQARRNRRRRWRERQR	+++	Futaki, 2005
		R7W	Fluo-RRRRRRRW-NH ₂	+++	Futaki, 2005
		TatP59W	Fluo-GRKKRRQRRRPWQ-NH ₂	+++	Futaki, 2005
		FHV Coat-(35-49)	RRRRNRTRNRRRV		Futaki, 2005
		BMV Gag-(7-25)	KMTRAQRRAAARRNR WTAR	+++	Futaki, 2005
		HTLV-II REX-(4-16)	TRRQRTRRARRNR	+++	Futaki, 2005
		CCMV Gag-(7-25)	KLTRAQRRAAARKNKR NTR	++	Futaki, 2005
		P22 N-(14-30)	NAKTRRHERRRKLAIER	++	Futaki, 2005
Basic	VP22 [HSV]	VP22	DAATATRGRSAASRPTE RPRAPARSASRPRPVE	+++	Cashman et al., 2002
	Human Calcitonin	hCT (9-32)	LGTYTQDFNKFHTFPQT AIGVGAP		Trehin et al., 2004
	Vascular endothelial cadherin [Mouse]	pVEC	LLIILRRRIRKQAHASK		Elmqvist et al., 2001
	Related to γ-Zein [Maize]	Sweet arrow peptide (SAP)	(VRLPPP) ₃		Fernandez-Carneado et al., 2004
	Related to γ-Zein [Maize]		(Tyr-ZnDPA) _n		Johnson et al., 2008
Basic/ amphiphilic	Antennapedia homeodomain [Drosophila]	Antennapedia (43-58) [penetratin]	RQIKIWFQNRRMKWKK	+++	Derossi et al., 1994
		Fluoro-Penetratin	Fluo-RQIKIWFQNRRMKWKK -NH ₂		Futaki, 2005

		Pen2W2F	Fluo- RQIKIFFQNRRMKFKK- NH ₂		Futaki, 2005
		Model amphipathic peptide	KLALKLALKALKAAALKL A-NH ₂		Futaki, 2005
		PenArg	Fluo- RQIRIWFQNRRMRWRR- NH ₂		Futaki, 2005
		PenLys	Fluo- KQIKIWFQNKKMKWKK -NH ₂		Futaki, 2005
		E N-(1-22)	MDAQTRRRERRAEKQA QWKAAN	+	Futaki, 2005
		B21 N-(12-29)	TAKTRYKARRAELIAER R	+	Futaki, 2005
		Yeast PRP6-(129- 144)	TRRNKRNRIQEQLNRK	+	Futaki, 2005
		Hum U2AF-(142- 153)	SQMTRQARRLYV	-	Futaki, 2005
Chimera (synthetic)	Galanin/ Mastoparan)	Transportan	GWTLNSAGYLLGKINLK ALAALAKKIL		Pooga et al., 1998
	Galanin/ Mastoparan)	Model amphipathic peptide (MAP)	KLALKLALKALKAAALKL A amide		Oehlke et al., 1998
	Hydrophobic- NLS	Pep-1	KETWWETWWTEWSQP KKKRKV		Morris et al., 2001
	Hydrophobic- NLS	CADY	Ac- GLWRALWRLRLSLWRL LWRA-cysteamide		Crombez et al., 2009
	Hydrophobic- NLS	Membrane translocating sequence peptide (MTS)	AAVALLPAVLLALLP		Rojas et al., 2008
	HIVGp41-NLS of SV40 T-antigen	MPG	GALFLGWLGAAGSTMG APKKKRKV		Morris et al., 2008

Table 3. Protein transduction domains (PTD)

Furthermore, the HIV Tat derived peptide is a small basic peptide that has been successfully shown to deliver a large variety of cargoes, from small particles to proteins, peptides and nucleic acids. The “transduction domain” or region conveying the cell penetrating properties is clearly confined to a small stretch of basic amino acids, with the sequence RKKRRQRRR (residues 49–57) [Riedl et al., 2004; Brooks et al., 2005]. This polycationic nanopeptide is known to be a transfection enhancer of plasmid DNA. The conditions of DNA-peptide complex formation and DNA/Tat ratio have significant impact on the level of transgene expression and degree of DNA protection from nuclease attack [Hellgren et al., 2004]. The conjugation of this peptide to ovalbumin (OVA) resulted in efficient stimulation of MHC class I-restricted T cell responses *in vitro* and, more importantly, the generation of CTLs *in vivo* [Kim et al., 1997]. Also, soluble Tat-antigen conjugates can deliver the antigen directly to the MHC class I processing pathway and thereby increase the generation of

antigen-specific CD8⁺ T cells *in vitro* [Kim et al., 1997; Riedl et al., 2004]. A fusion protein containing the carboxy-terminal end of Tat (amino acids: 49–86) linked to the HPV16 E7 oncoprotein enhanced tumor specific immune responses *in vivo* [Giannouli et al., 2003]. In C57BL/6 mice, E7-Tat mixed with Quil A generated efficient prophylactic and therapeutic suppression of HPV16-positive C3 tumor outgrowth. This study offers a new strategy for improving subunit cancer vaccines [Giannouli et al., 2003]. Particularly, a Tat-derived peptide in combination with a PEG-PEI copolymer could be a promising candidate as gene delivery vehicle intended for pulmonary administration. Tat-PEG-PEI represents a new approach to non-viral gene carrier for lung therapy, comprising protection for plasmid DNA, low toxicity and significantly enhanced transfection efficiency under *in vivo* conditions [Kleemann et al., 2005].

It has been shown that covalent attachment of low molecular weight polyethyleneimine (PEI) improves Tat peptide mediated gene delivery *in vitro* [Alexis et al., 2006; Putnam et al., 2001; Wang, 2006]. In our recent study, two delivery systems including polymer PEI 25 kDa and polymer peptide hybrid as PEI600-Tat conjugate were used to compare their efficiency for HPV16 E7 DNA transfection *in vitro*. Our data indicated that both delivery systems including PEI 25 kDa and PEI600-Tat conjugate are efficient tools for E7 gene transfection. In fact, PEI potency for E7 gene transfection is higher than PEI600-Tat *in vitro*, but its toxicity is obstacle *in vivo* [Bolhassani et al., 2008]. Using HPV16 E7 as a model antigen, the effect of PEI600-Tat conjugate has been evaluated on the potency of antigen-specific immunity in mice model. Assessment of lymphoproliferative and cytokine responses against recombinant E7 protein (rE7) showed that PEI600-Tat/E7DNA complex at certain ratio induces Th1 response. This study has demonstrated that PEI600-Tat conjugate is efficient to improve immune responses *in vivo* [Bolhassani et al., 2009].

Moreover, synthetic peptides containing a nuclear localization signal (NLS) can be bound to the DNA and the resulting DNA-NLS complexes can be recognized as a nuclear import substrate by specific intracellular receptor proteins [53]. For example, conjugation of an NLS to a Minimalistic Immunogenically Defined Gene Expression (MIDGE) vector encoding a truncated and secreted form of BHV-1 glycoprotein D (tgD) improved the tgD expression *in vitro* and induced both humoral and cellular immune responses in mice [Zheng et al., 2006]. This strategy could be applied as an efficient pathway in enhancement of DNA vaccine potency against cancer.

On the other hand, one of the CPPs that have currently received extensive attention in the field of DNA vaccination is the herpes simplex virus (HSV-1) protein VP22 [Brooks et al., 2010]. VP22 can form compacted complexes with short oligonucleotides and form particles of spherical nature with a size range of 0.3 to 1 μm in diameter. These particles entered cells efficiently within 2 to 4 hours. Furthermore, VP22 enables spreading of the antigenic peptide to the cells surrounding the transfected cells [Brooks et al., 2010]. Efforts have been made to increase the potency of DNA vaccines by exploiting the cell-to-cell spreading capabilities of the HSV-1 VP22 protein or the analogous protein from bovin herpesvirus 1 [Ulmer et al., 2006]. The significance of VP22 in intercellular spreading has been demonstrated through *in vitro* studies linking VP22 to p53, thymidine kinase, cytosine deaminase and Green Fluorescent Protein (GFP). These proteins were observed to be distributed to nuclei of surrounding cells [Lin et al., 2010]. Furthermore, vaccination with DNA encoding HPV16E7 linked to the HSV type 1 VP22 elicited the enhanced E7-specific memory CD8⁺ T lymphocytes and anti-tumor effects against E7-expressing tumor cells [Michel et al., 2002]. Also, VP22 has been used for HPV DNA vaccines targeting the

E6 protein [Lin et al., 2010]. Various groups have demonstrated that DNA constructs which encode fusion proteins of VP22 linked to an antigen increase the immune responses in mice and cattle. Bovine herpesvirus VP22 (BVP22) and Marek's disease virus VP22 (MVP-1) are both closely related by their structural homology to HSV-1 VP22, and can also have a significant role in intercellular spreading. Hung *et al.* has demonstrated that mice vaccinated with DNA encoding MVP22/E7 significantly increased numbers of IFN- γ -secreting, E7-specific CD8⁺ T cell precursors compared to mice vaccinated with wild-type E7 DNA alone, which directly lead to a stronger tumor prevention response. Similarly, immunization of mice and cattle with DNA vaccine coding for BVP22 linked to truncated glycoprotein D (BVP-tgD) was shown to generate a stronger tgD-specific immune response compared to animals vaccinated with tgD alone. Taken together, DNA vaccine encoding VP22 linked to antigens represents a promising approach to enhance DNA vaccine potency [Lin et al., 2010].

To evaluate the VP22 role in gene therapy of hepatocellular carcinomas (HCCs), the expression vectors were constructed for N- and C-terminal fragments of VP22-p53 fusion proteins and investigated the VP22-mediated shuttle effect in hepatoma cells by co-transfection experiments. VP22-mediated trafficking was not detectable in hepatoma cells *in vitro* by fluorescence microscopy [Zender et al., 2002]. For *in vivo* experiments, the recombinant adenoviruses Ad5CMVp53 and Ad5CMVp53-VP22 were constructed. In contrast to the *in vitro* experiments, intercellular trafficking of VP22-p53 could be observed in subcutaneous tumors of hepatoma cells by fluorescence microscopy, indicating a stronger shuttle effect in solid tumors compared to cell culture experiments [Zender et al., 2002]. In our current study, Herpes simplex virus type 1 (HSV-1) VP22 protein was employed to enhance DNA vaccine potency of *Leishmania major* amastin antigen in BALB/c mice model. Vaccination with the VP22-amastin-EGFP fusion construct elicited significantly higher IFN- γ response upon antigen stimulation of splenocytes from immunized mice compared to amastin as a sole antigen. These results suggest that the development of DNA vaccines encoding VP22 fused to a target *Leishmania* antigen would be a promising strategy to improve immunogenicity and DNA vaccine potency [Bolhassani et al., 2011].

7. Hybrid vectors

A promising approach to overcome the limitations and develop the advantages of the individual types of vectors is their combination. Several types of hybrid vectors have been known: A) Virosomes are produced by the fusion of lipoplexes (liposomes with DNA) with inactivated HVJ virus (hemagglutinating virus of Japan) or influenza virus [Gardlik et al., 2005]. It was shown that the efficiency of gene transfer into the respiratory tract is higher than cationic liposomes or viral vectors. In addition, they are very well tolerated from the immunological view, so even repeated injection does not influence the efficiency and safety of transfer; B) The second type is represented by hybrids that were generated by mixing cationic liposomes or polymers with adenoviral vector. These are effective mainly in cells which do not have viral receptors. In addition, it was proved that an inactivated adenovirus attachment improves the efficiency of the transfer mediated by cationic liposomes or polymers [Gardlik et al., 2005]; C) Hybrid viruses can be produced by a combination of various types of viral vectors, and they represent a system which employs the main advantages of both viruses [Gardlik et al., 2005].

8. Bacterial delivery systems

The most recent approach in targeting the gene therapy is the use of bacterial systems as vectors for transfer and gene expression in tumor cells. The principle lies in the ability of some anaerobic bacteria to selectively colonize hypoxic areas (e.g., tumors) and replicate there. Therefore, it is possible to achieve selective expression of therapeutic genes specifically in tumors. Currently known and tested bacterial vectors have been divided into two groups: A) Strictly anaerobic bacteria (the species *Clostridium* and *Bifidobacterium*) are used in *in vivo* experiments. *Clostridium* is the most important bacterial species for use as a vector. On the other hand, the non-pathogenic *Bifidobacterium*, is naturally present in the human gastrointestinal tract and provides higher safety [Gardlik et al., 2005]. B) The second group consists of attenuated auxotrophic strains of *Salmonella typhimurium* that require the presence of tumor specific nutrition factors for selective replication. They use these factors for their own metabolism, thus prohibiting the tumor cells from utilizing them and growing. In *in vivo* experiments, high levels of *Salmonella* (10⁹ bacteria/g tissue) were obtained. A similar tumor inhibitory effect was shown by application of *Salmonella* producing thymidine-kinase [Gardlik et al., 2005]. Although the above bacteria are characterized by high selectivity in tissue colonization, it is necessary to ensure the maximum level of specificity and therapeutic efficiency of bacterial vectors. For this purpose, promoters inducible by radiation were constructed. The transcription of such promoters is conditioned by irradiation with visible light. Bacteria are known for their resistance to irradiation (having a relatively small genome and effective DNA repair mechanisms). Therefore the localization of a therapeutic gene under the control of a radiation-inducible promoter ensures that cytotoxic proteins are expressed only in bacteria colonizing currently irradiated tissues. This strategy allows eliminating the possibility of expression in non-tumor and hypoxic tissues. The latest approach using prokaryotes in gene therapy is a system of transformed bacteria producing a therapeutic protein *in situ* under exogenous induction regulation [Gardlik et al., 2005].

9. Eukaryotic delivery systems

9.1 *Leishmania tarentolae* as a novel live vector

Although live recombinant vectors (bacterial or viral recombinant vectors) have been known to develop new vaccine strategies against pathogens (e.g., HIV-1), their use as vaccine candidates in human is delayed due to problems related to pre-existing immunity, inefficient antigen delivery or presentation and toxicity issues. Therefore, it is necessary to develop new live-vaccine vectors that are able to enhance antigen presentation and elicit potent immune responses without the risk of developing disease in humans. Recently, a lizard parasitic protozoan that is not pathogenic to humans, *Leishmania tarentolae* (*L. tarentolae*), has been used as a candidate vaccine against visceral leishmaniasis [Breton et al., 2005], and HIV-1 [Breton et al., 2007]. *L. tarentolae* can elicit T-cell proliferation and the production of gamma interferon (IFN- γ), skewing the T-cell response towards a Th1-cell phenotype, and it provides inflammatory responses for the APC and acts as an immunostimulatory adjuvant. Unlike other pathogenic *Leishmania* strains, *L. tarentolae* lacks the potential to replicate within the targeted APCs and is eliminated after several days from the infected murine host [Breton et al., 2005; Breton et al., 2007]. It has been shown that a single intraperitoneal injection of *L. tarentolae* could elicit a protective immune response against infectious challenge with *L. donovani* in susceptible BALB/c mice [Breton et al., 2005]. Similarly, a single intraperitoneal administration of the A2-recombinant *L. tarentolae*

strain could induce high levels of IFN-gamma and protect BALB/c mice against *L. infantum* challenge [Mizbani et al., 2009]. Interestingly, a recombinant *L. tarentolae* vaccine expressing high levels of full-length HIV-1 Gag elicited cell-mediated immunity in mice model and decreased HIV-1 replication in human tonsillar tissue following exposure to HIV-1 infection [Breton et al., 2007]. These data suggest that the use of *L. tarentolae* as a live vaccine vector may represent a promising approach for improving immunity and safety of candidate live vaccines against *Leishmania* infections and likely other intracellular pathogens for which T-cell mediated responses are critical for the development of protective immunity [Breitling et al., 2002; Breton et al., 2005].

9.2 Yeast as an efficient tool in vaccine development

Recent studies have indicated that yeast cell wall components possess multiple adjuvant properties. Interactions between yeast and DCs result in DC maturation, and whole recombinant yeast internalized by DCs can deliver heterologous antigens to both MHC class I and class II pathways and induce potent cell-mediated immunity [Capilla et al., 2009; Bian et al., 2010; Haller et al., 2007]. Vaccination with *Saccharomyces cerevisiae* (*S. cerevisiae*) expressing tumor-associated antigens can induce antigen-specific T-cell responses and protect animals against tumor challenge. In addition, *S. cerevisiae* is inherently nonpathogenic and heat-killed recombinant *S. cerevisiae* shows no toxicity in clinical studies. Yeast can be easily engineered to express multiple antigens and the inherent adjuvant properties of *S. cerevisiae* avoid the need for additional adjuvants. These characteristics make *S. cerevisiae* a potential vaccine vehicle for cancer and infectious diseases [Capilla et al., 2009; Bian et al., 2010; Haller et al., 2007]. There are some limitations and drawback in *S. cerevisiae* expression systems. For example, *S. cerevisiae* has a tendency to hyperglycosylate recombinant proteins, N-linked carbohydrate chains are terminated with alpha-1, 3-linked mannose residues which is considered to be allergenic. Other restriction is that the varieties of carbon sources that can be utilized by this species are limited [Bian et al., 2010]. Currently, two other species including *Hansenula polymorpha* and *Pichia pastoris* belonging to the *Saccharomycetaceae* family, could potentially overcome the described limitations of *S. cerevisiae* [Bazan et al., 2009; Bian et al., 2010]. On the other hand, these two species are broadly used as industrial platforms for heterologous protein production [Maleki et al., 2010; Bian et al., 2010].

10. Conclusion

A number of methods have been and are being invented for the efficient and safe delivery of therapeutic DNA. The perspectives and hopes that are associated with gene therapy support research in this field of molecular biology. Although, clinical trials have already started, there are still various limitations that must be solved before routine clinical use. The major aim in gene therapy is to develop efficient, non-toxic gene carriers that can encapsulate and deliver foreign genetic materials into specific cell types including cancerous cells. Both viral and non-viral vectors were developed and evaluated for delivering therapeutic genes into cancer cells. Many viruses such as *retrovirus*, *adenovirus*, *herpes simplex virus*, *adeno-associated virus* and *pox virus* have been modified to eliminate their toxicity and maintain their high gene transfer capability. Due to the limitations correlated to viral vectors, non-viral vectors have been further focused as an alternative in delivery systems. The main non-viral vectors include cationic polymers, cationic peptides and cationic liposomes. Currently, many modifications to the current delivery systems and novel carrier systems have been

developed to optimize the transfection efficiency. Furthermore, the route of immunization can influence the outcome of the immune response through altering the interaction between the vaccine and different APCs at the site of injection. Hence, the routes of administration and formulation of DNA clearly affect the therapeutic response by altering immune pathway. Among the commonly used methods of DNA vaccination, the highest efficacy was achieved after *in vivo* electroporation and gene gun delivery. However, it is critical to further analyze the results of ongoing clinical trials, specifically, in the aspect of their success or failure of certain delivery methodologies for gene therapies.

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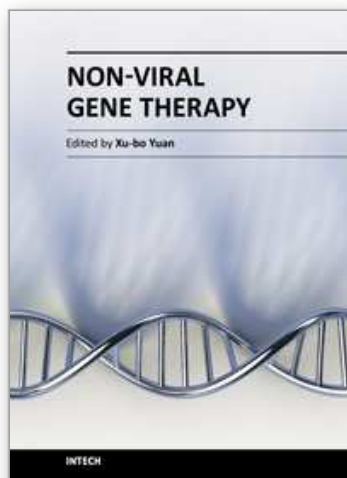
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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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